Proc. Natl. Acad. Sci. USA
Vol. 75, No. 5, pp. 2315–2319, May 1978
Cell Biology

Carbohydrate structure and cell differentiation: Unique properties of fucosyl-glycopeptides isolated from embryonal carcinoma cells

(glycopeptides/teratocarcinoma/mouse embryo/cell surface)

T. MURAMATSU, G. GACHELIN, J. F. NICOLAS, H. CONDAMINE, H. JAKOB, AND F. JACOB

Service de Génétique cellulaire du Collège de France et de l'Institut Pasteur, 25, rue du Dr. Roux, 75015, Paris, France

Contributed by François Jacob, February 13, 1978

ABSTRACT **From embryonal carcinoma cells labeled with fucose, two main classes of glycopeptide products of Pronase digestion can be distinguished by Sephadex G-50 column chromatography: one eluted near the excluded volume and a smaller one. The large fucosyl-glycopeptides are scarcely present in differentiated cells derived from embryonal carcinoma cells (i.e., fibroblastlike cells, myoblasts, and parietal yolk-sac carcinoma). During *in vitro* differentiation of embryonal carcinoma cells, these large glycopeptides disappear almost completely. The small glycopeptides were analyzed by paper electrophoresis, concanavalin A-Sepharose affinity chromatography, and digestion with an endoglycosidase. The major components of these glycopeptides from embryonal carcinoma cells appear to be different from complex glycopeptides known to occur in adult cells. The glycopeptide pattern of mouse preimplantation embryos resembles that of embryonal carcinoma cells. These results suggest that the carbohydrate profile changes fundamentally during early stages of mammalian development.**

Mouse teratocarcinoma is especially useful for studying certain aspects of early mammalian development. The stem cells of teratocarcinoma, known as embryonal carcinoma (EC) cells, resemble those of early embryos and can differentiate into a variety of cell types (1, 2). EC cells can be obtained in large amounts, and thus provide a suitable material for biochemical and immunological analysis. Immunological studies of EC cells have already allowed the detection of cell surface antigens common to normal early embryonic cells (3).

This communication deals with differences between carbohydrate moieties of glycoproteins from EC cells and those from EC-derived differentiated cells. Carbohydrate moieties of glycoproteins are assumed to play an important role in cellular differentiation through cell surface interactions. Significant differences in the number of receptor sites for various lectins have already been observed between EC and differentiated cells, as well as during *in vitro* differentiation of EC cells (4, 5). Thus, carbohydrate moieties of glycoproteins may be expected to undergo significant alterations during the early stages of mammalian development.

MATERIALS AND METHODS

Cell Culture and Labeling with Radioactive Fucose. EC cells and EC-derived differentiated cells (Table 1) were cultured in Falcon tissue culture dishes in Dulbecco's modified Eagle's medium containing 15% fetal calf serum at 37° in an atmosphere of 12% CO_2 (8). For $F9_{41}$ cells, the dishes were coated with gelatin (6). For PYS-2 cells, only 5% fetal calf serum was used. All cells were routinely tested for mycoplasma contamination; none was found. For labeling with radioactive sugars, the cells were plated in 10-cm tissue culture dishes. After 24 hr, when the cells reached exponential growth phase, 50 μCi of [6-^{3}H]fucose (10–25 Ci/mmol, Commissariat à l'Energie Atomique) was added to each dish. The cells were cultured for 24 hr, washed once with Earle's solution lacking Mg^{2+} and Ca^{2+}, and harvested from tissue culture dishes by EDTA treatment (6). With PCC4/F and PCC4/AzaR1, the cells were harvested by gentle pipetting (8). The harvested cells were washed once with Dulbecco's modified Eagle's medium and stored at −20°.

Table 1. Relationship between EC cells and EC-derived differentiated cells

Origin	Cell lines: EC cells	Cell lines: Differentiated cells
129/SV mice (teratocarcinoma OTT6050)	Nullipotent cells $F9_{41}$ (6)	Parietal yolk-sac carcinoma PYS-2 (7)
	Multipotent cells PCC4/AzaR1 (8)	
	PCC4F (8)	
	PCC3/A/1 (9) →	Fibroblastlike cells PCC3/A/1-D3* (Nicolas, unpublished)
C3H mice (teratocarcinoma C_{17})	Multipotent cells $C_{17}S_1$ clone 1003† (10) →	Myoblasts $C_{17}S_1$-T984 (11)

Arrows indicate that the differentiated cells were derived from the line of EC cells. Numbers in parentheses are references.
* Retained diploid karyotype, morphologically similar to fibroblasts.
† Recloned without feeder layer.

Pronase Digestion and Gel Filtration. Harvested cells (1–3 × 10^7 cells) were incubated with 40 mg of Pronase (Calbiochem, B grade) in 4 ml of 0.05 M Tris·HCl, pH 8.0, containing 0.01 M $CaCl_2$ at 37° for 24 hr under a toluene layer. Forty milligrams of Pronase in 2 ml of reaction medium was added after 24 hr and 48 hr, and the digestion was terminated after 72 hr. Embryos were digested in a similar way, except that the volume of the reaction mixture was reduced to 1/40. The digested material was centrifuged; the supernatant was concentrated by lyophilization and applied to a column of Sephadex G-50, superfine (1.7 × 62 cm) that had been equilibrated and was eluted by 0.05 M acetic acid buffered with ammonium hydroxide to pH 6.0. Glycopeptide standards used to calibrate the column were prepared as described previously (12).

Other Methods. Radioactivity was measured in an Intertechnique SL30 liquid scintillation counter. Unisolve 1 (Koch–Light Laboratories) was used as scintillation fluid. Digestion with endo-β-*N*-acetylglucosaminidase D (endo D) was carried out in the presence of neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase as described previously (12).

Abbreviations: EC, embryonal carcinoma; endo D, endo-β-*N*-acetylglucosaminidase D; Con A, concanavalin A.

RESULTS

Elution Profile of Fucosyl-Glycopeptides upon Sephadex G-50 Column Chromatography. Exponentially growing EC cells and EC-derived differentiated cells (Table 1) were labeled with [^{3}H]fucose. This sugar was selected as the initial probe because it is known to be incorporated into a variety of cells without being converted to other sugars (13–15). This point was confirmed by analyzing fucose-labeled glycopeptides (see below) from EC cells and EC-derived cells by acid hydrolysis and paper chromatography as described earlier (16). Furthermore, fucosyl-glycoproteins are known to be mostly located in plasma membranes, at least in HeLa cells and fibroblasts (17, 18). This point was also confirmed in a line of EC cells, $F9_{41}$, by isolation of plasma membranes from the cells (T. Muramatsu, G. Gachelin, and F. Jacob, unpublished results).

The fucose-labeled cells were extensively digested with Pronase, and the resulting glycopeptides were analyzed by Sephadex G-50 column chromatography (Fig. 1). Two distinct components were observed in the eluate of glycopeptides from EC cells: high-molecular-weight glycopeptides and low-molecular-weight glycopeptides (Fig. 1 *A*, *B*, and *C*).

Large Glycopeptides. In all EC cell lines examined (Table 1), significant amounts of fucosyl-glycopeptides were eluted near the excluded volume of the column. This applies to both nullipotent cells, which have lost the capability of differentiation (Fig. 1*A*), and multipotent cells, which have retained the capability of differentiation (examples shown in Fig. 1 *B* and *C*). Most EC cells examined were derived from teratocarcinoma OTT 6050 of mouse line 129 (Table 1); however, a line derived from a C3H teratocarcinoma was also found to contain the large glycopeptides (Fig. 1*C*).

In contrast, the large glycopeptides were scarcely present in EC-derived differentiated cells: parietal yolk-sac carcinoma PYS-2 (Fig. 1*D*), fibroblastlike cells (Fig. 1*E*), and myoblasts (Fig. 1*F*).

The large glycopeptides found in EC cells differ from the transformation-dependent and/or growth-dependent glycopeptides described in a variety of malignant cells (14, 15). The latter glycopeptides are smaller (19) and should be eluted in fractions 50–60 in the present chromatographic system (Fig. 1).

The high-molecular-weight nature of the large glycopeptides from EC cells is not due to incomplete Pronase digestion, because redigestion of the isolated large glycopeptides did not change the elution profile. Furthermore, less than 0.05% of an amino acid mixture incorporated into EC cells was recovered in the large glycopeptides. Alkaline treatment (0.2 M NaOH with 0.4 M $NaBH_4$ at 25° for 24 hr) did not depolymerize the glycopeptides, although the above treatment is expected to release significant amounts of oligosaccharides *O*-glycosidically linked to polypeptide backbone. Extraction with chloroform/methanol (2:1) did not remove any radioactivity. The large glycopeptides could also be labeled by feeding the cells with radioactive galactose or glucosamine. Detailed biochemical properties of the glycopeptides will be described elsewhere (T. Muramatsu, G. Gachelin, and F. Jacob, unpublished results).

The presence of the large glycopeptides was investigated during *in vitro* differentiation of PCC3/A/1 cells (2, 9). In the

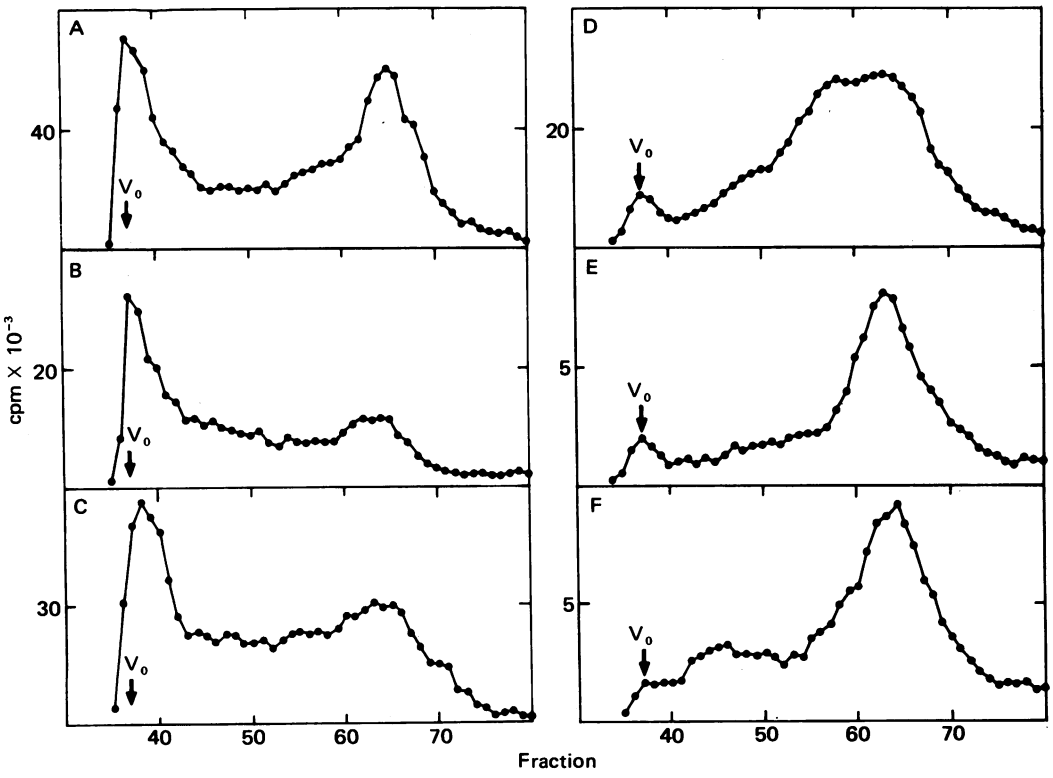

FIG. 1. Analysis of fucose-labeled glycopeptides from EC cells and EC-derived differentiated cells by Sephadex G-50 column chromatography. [^{3}H]Fucose-labeled cells (approximately 3×10^7 cells in experiments A, B, C, and D, and approximately 1×10^7 cells in experiments E and F) were extensively digested with Pronase and the hydrolysate was analyzed on a column of Sephadex G-50, superfine (1.7 × 62 cm). Fractions (1.7 ml) were collected. Standards—blue dextran, [^{14}C]acetylated fetuin glycopeptide (molecular weight around 3500), [^{14}C]acetylated IgG glycopeptide (molecular weight around 2000), and glucose—were eluted in fractions 36–38, 58–59, 67–68, and 88–91, respectively. An aliquot of each fraction was analyzed for radioactivity. Results were expressed as total amounts of radioactivity per each fraction. V_0, excluded volume. (*A*) $F9_{41}$; (*B*) PCC4F; (*C*) $C_{17}S_1$ clone 1003; (*D*) PYS-2; (*E*) PCC3A/1-D3; and (*F*) $C_{17}S_1$-T984.

initial cell population, a significant amount of the large glycopeptides was detected (Fig. 2*A*). However, as the cells were allowed to differentiate in the dishes, the amount of large glycopeptides decreased dramatically. On day 14 of differentiation, only slight amounts of the large glycopeptides were found (Fig. 2*B*). They disappeared almost completely on day 28 of differentiation (Fig. 2*C*), a time when only 0.1% of the population is EC cells while a variety of differentiated cells have appeared (2, 9).

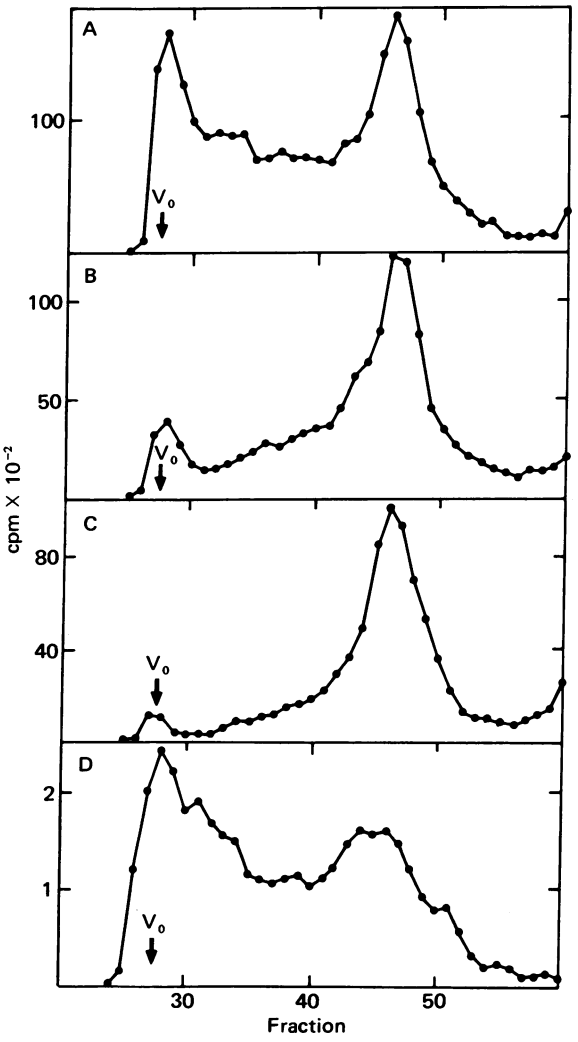

FIG. 2. Comparative analysis of fucose-labeled glycopeptides from PCC3/A/1 cells during the process of *in vitro* differentiation and from mouse preimplantation embryo. Confluent cultures of PCC3/A/1 cells were allowed to differentiate *in vitro* according to the procedure of Nicolas *et al.* (9). On day 14 or day 28, the cells were dissociated by trypsin and replated. After 24 hr, the cells were fed with [^{3}H]fucose and cultured for another 24 hr. Mouse embryos (two-cell stage) were obtained from superovulated virgin C57BL/6 females (5 international units of pregnant mare serum injected intraperitonally, followed 40 hr later by the injection of 5 international units of human chorionic gonadotropin), mated with F_1 C57BL/6 × CBA males. The embryos were cultured in Whitten's medium (20) containing [^{3}H]fucose at 20 μCi/ml. After 48 hr, 7 embryos were fully expanded blastocysts, 2 were young blastocysts, and 38 were late morulae. The embryos were pooled and washed four times with Whitten's medium. EC cells or embryos were extensively digested with Pronase, and the digests were analyzed by Sephadex G-50 column chromatography. Fractions (2.3 ml) were collected. Blue dextran and glucose were eluted in fractions 27–28 and 65–68, respectively. (*A*) PCC3/A/1 at day 0; (*B*) PCC3/A/1 at day 14; (*C*) PCC3/A/1 at day 28; and (*D*) mouse embryos.

Table 2. Comparative analysis of the small glycopeptides from EC cells and EC-derived differentiated cells

Source	% of glycopeptides		
	Acidic*	Bound to Con A-Sepharose†	Hydrolyzed by endo D‡
$F9_{41}$ (nullipotent EC cells)	36.0	34.9	20.4
PCC4F (multipotent EC cells)	42.3	46.7	36.1
PYS-2 (parietal yolk-sac carcinoma)	44.0	70.1	37.2
$C_{17}S_1$-T984 (myoblasts)	61.3	76.3	62.5
PCC3/A/1-D3 (fibroblastlike cells)	65.6	74.0	63.4

Glycopeptides eluted in fractions 60–75 (Fig. 1) were pooled and analyzed by three different methods.

* Paper electrophoresis at pH 6.5 (16).

† Affinity-column chromatography on concanavalin A-Sepharose (21). Glycopeptides bound to the column were mostly recovered by elution with 0.1 M methyl α-mannoside, and trace amounts remaining attached to column were recovered by 1% sodium dodecyl sulfate (12, 21).

‡ Hydrolysis by endo D in the presence of neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase. The products were analyzed by Sephadex G-25 column chromatography in order to determine the amount of low-molecular-weight products, Fuc-GlcNAc-peptides (12).

The presence of the large fucosyl-glycopeptides was investigated in cultured mouse embryos. Two-cell-stage embryos were collected from mice and cultured for 48 hr with [^{3}H]-fucose. When they were harvested, most of the embryos had reached the late morula stage. The large glycopeptides were found to represent a major component of the glycopeptides from the embryos (Fig. 2*D*).

Small Glycopeptides. Upon Sephadex G-50 column chromatography, significant amounts of fucosyl-glycopeptides were eluted in the low-molecular-weight region (fractions 60–75 in Fig. 1), with both EC cells and EC-derived differentiated cells. Molecular weights of the small glycopeptides were estimated to be around 3000–2500 in their peak position (fractions 63–65, Fig. 1) by calibrating the column with glycopeptide standards. Thus with respect to size characteristics, these small glycopeptides were similar to the major fucosyl-glycopeptides found in normal fibroblasts, which are known to have the structure shown in Fig. 3 (12). A detailed investigation was therefore carried out to determine whether or not the small glycopeptides from EC cells and EC-derived differentiated cells conform to that general structure. The following three methods of analysis were used (Table 2).

(*i*) Separation of neutral glycopeptides and acidic glycopeptides by paper electrophoresis. If the fucosyl-glycopeptides are sialylated as in the case of the major glycopeptides from fibroblasts, they should behave as acidic glycopeptides.

(*ii*) Fractionation of glycopeptides by affinity column chromatography on Con A-Sepharose. If the glycopeptides have more than two α-mannosyl residues that are not substituted at the hydroxyl groups linked to C-3, C-4, and C-6, they are retained by the column (21). This is known for the major fucosyl-glycopeptides from fibroblasts.

(*iii*) Hydrolysis of the glycopeptides by endo-β-*N*-acetylglucosaminidase D (endo D) (22). Most fucosyl-glycopeptides from fibroblasts are known to be hydrolyzed by endo D in the presence of exoglycosidases that remove peripheral sugars from the glycopeptides. After the reaction, labeled fucose is recov-

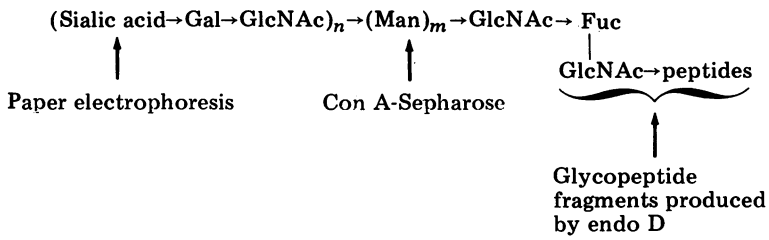

FIG. 3. Proposed structure of the major components of fucosyl-glycopeptides from cultured fibroblasts (12). Vertical arrows indicate the particular features detected with the three analytical procedures used in Table 2. $n = 2$ or 1; $3 \leq m \leq 6$. Con A, concanavalin A.

ered attached to small fragments, Fuc-GlcNAc-peptides, because in fibroblasts fucose is mainly linked to *N*-acetylglucosamine involved in protein-carbohydrate linkage (12, 16).

By the three criteria, the small glycopeptides from EC cells were found to differ significantly from those of fibroblasts (Table 2). The major components of the small glycopeptides from EC cells were (*i*) neutral species, (*ii*) unadsorbed to Con A, and (*iii*) resistant to endo D. In contrast, the major fucosyl-glycopeptides from two lines of EC-derived differentiated cells (myoblasts and fibroblastlike cells) had properties very similar to those of the major fucosyl-glycopeptides from fibroblasts (Table 2). The major component of fucosyl-glycopeptides from parietal yolk-sac carcinoma (PYS-2) were similar to those of other differentiated cells in terms of their reactivity with Con A-Sepharose, while they were different in terms of the other two properties (Table 2). Parietal yolk-sac cells belong to a differentiation path different from fibroblasts and myoblasts in the development of the mouse embryo. It is thus tempting to speculate that the intermediate properties of the fucosyl-glycopeptides of parietal yolk-sac carcinoma are related to the developmental sequence.

DISCUSSION

Fucose-labeled glycopeptides of EC cells were found to be strikingly different from those of EC-derived differentiated cells. The most prominent characteristic of the glycopeptides from EC cells was the abundance of the material eluted near the excluded volume upon Sephadex G-50 column chromatography. These glycopeptides were scarcely present in EC-derived differentiated cells, and they disappeared almost completely during *in vitro* differentiation of EC cells. They were also present in preimplantation stage embryos. That these glycopeptides are mainly derived from the cell surface of EC cells has been confirmed by two other observations: (*i*) glycopeptides prepared from cell surface material released by mild trypsin digestion also contained a significant amount of the large glycopeptides; (*ii*) plasma membrane glycopeptides purified by $F9_{41}$ cells were very similar to the whole cell glycopeptides (T. Muramatsu, G. Gachelin, and F. Jacob, unpublished results). Fucosyl-glycopeptides with such a high molecular weight have not been reported to occur in significant amounts in whole cells, plasma membranes, or cell surface material of a number of normal and malignant cells; i.e., normal and virus-transformed fibroblasts (12, 14, 19), liver cells (15), hepatoma cells (15), lymphoma cells (15, 23), neuroblastoma cells (24), and Meth-A fibrosarcoma cells (T. Muramatsu and S. G. Nathenson, unpublished results). Thus, the abundance of the large fucosyl-glycopeptides in EC cells so far appears to be correlated with the early embryonic phenotype of the cells. This is supported by the finding that human embryonal carcinoma cells Susa (25) labeled with fucose display a similar pattern (T. Muramatsu, G. Gachelin, and F. Jacob, unpublished results).

Another interesting characteristic of fucosyl-glycopeptides from EC cells was that the major components of the low-molecular-weight fraction were different from typical complex glycopeptides (26). The usual adult complex sugar chains are major components of carbohydrate moieties of serum glycoproteins (26), and of cell surface glycoproteins such as H-2 alloantigens (23), and constitute the major fucosyl-glycopeptides from fibroblasts (12). Low-molecular-weight glycopeptides from polyoma-transformed baby hamster kidney (BHK) cells also appear to have the same structure (19). The results described in this paper indicate that major fucosyl-glycopeptides from myoblasts have the same structure as well. Therefore, the unique properties of small glycopeptides from EC cells may again be correlated with their undifferentiated phenotype, although additional information about the properties of the small glycopeptides from other differentiated cells is required before definitive conclusions can be drawn.

The present finding of significant changes in the carbohydrate moieties of glycoproteins during differentiation of early embryonic cells is important for understanding the mechanism of cell differentiation. First of all, the results clearly indicate that specific glycopeptides, glycoproteins, and glycosyltransferases can be used as markers of cell differentiation. Second, carbohydrate chains characteristic of some developmental stages are expected to play a critical role in cell interactions during embryogenesis. The large fucosyl-glycopeptides found in EC cells are of special interest in this respect.

T.M. has been on leave of absence from Kobe University School of Medicine, and was supported by the Franco-Japanese exchange program between l'Institut National de la Santé et de la Recherche Médicale and the Japan Society for the Promotion of Science. This work was supported by grants from the Centre National de la Recherche Scientifique, l'Institut National de la Santé et de la Recherche Médicale, the Délégation Générale à la Recherche Scientifique et Technique, the National Institutes of Health, and the André Meyer Foundation.

1. Martin, G. R. (1975) *Cell* **5**, 229–243.
2. Nicolas, J. F., Avner, P., Gaillard, J., Guénet, J. L., Jakob, H. & Jacob, F. (1976) *Cancer Res.* **36**, 4224–4231.
3. Jacob, F. (1977) *Immunol. Rev.* **33**, 3–32.
4. Gachelin, G., Buc-Caron, M. H., Lis, H. & Sharon, N. (1976) *Biochim. Biophys. Acta* **436**, 825–832.
5. Reisner, Y., Gachelin, G., Dubois, P., Nicolas, J. F., Sharon, N. & Jacob, F. (1977) *Dev. Biol.* **61**, 20–27.
6. Artzt, K., Dubois, P., Bennett, D., Condamine, H., Babinet, C. & Jacob, F. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2988–2992.
7. Lehman, J. M., Speers, W. C., Swartzendruber, D. E. & Pierce, G. B. (1974) *J. Cell. Physiol.* **84**, 13–28.
8. Jakob, H., Boon, T., Gaillard, J., Nicolas, J. F. & Jacob, F. (1973) *Ann. Microbiol. (Paris)* **124B**, 269–282.
9. Nicolas, J. F., Dubois, P., Jakob, H., Gaillard, J. & Jacob, F. (1975) *Ann. Microbiol. (Paris)* **126A**, 3–22.
10. McBurney, M. W. (1976) *J. Cell. Physiol.* **89**, 441–455.
11. Jakob, H., Buckingham, M. E., Cohen, A., Dupont, L., Fiszman, M. & Jacob, F. (1978) *Exp. Cell Res.*, in press.
12. Muramatsu, T., Ogata, M. & Koide, N. (1976) *Biochim. Biophys. Acta* **444**, 53–68.
13. Kaufman, R. L. & Ginsburg, V. (1968) *Exp. Cell Res.* **50**, 127–132.

14. Buck, C. A., Glick, M. C. & Warren, L. (1970) *Biochemistry* **9**, 4567–4576.
15. Beek, W. P., Smets, L. A. & Emmelot, P. (1973) *Cancer Res.* **33**, 2913–2922.
16. Muramatsu, T., Atkinson, P. H., Nathenson, S. G. & Ceccarini, C. (1973) *J. Mol. Biol.* **80**, 781–799.
17. Atkinson, P. H. & Summers, D. F. (1971) *J. Biol. Chem.* **246**, 5162–5175.
18. Gahmberg, C. G. (1971) *Biochim. Biophys. Acta* **249**, 81–95.
19. Ogata, S., Muramatsu, T. & Kobata, A. (1976) *Nature* **259**, 580–582.
20. Whitten, W. K. & Biggers, J. D. (1968) *J. Reprod. Fertil.* **17**, 399–401.
21. Ogata, S., Muramatsu, T. & Kobata, A. (1975) *J. Biochem. (Tokyo)* **78**, 687–696.
22. Koide, N. & Muramatsu, T. (1974) *J. Biol. Chem.* **249**, 4897–4904.
23. Muramatsu, T. & Nathenson, S. G. (1970) *Biochemistry* **9**, 4875–4883.
24. Glick, M. C., Kimhi, Y. & Littauer, U. Z. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 1682–1687.
25. Hogan, B., Fellous, M., Avner, P. & Jacob, F. (1977) *Nature* **270**, 515–518.
26. Kornfeld, R. & Kornfeld, S. (1976) *Annu. Rev. Biochem.* **45**, 217–237.